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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Dynamic Genetic Control:
Manipulating Cell Behaviors Using Heat

A thesis submitted in partial satisfaction of the
requirements for the degree
Master of Science

in

Bioegnineering

by

Yanmin Ji

Committee in charge:

Professor Peter Yingxiao Wang, Chair
Professor Nan Hao
Professor Prashant Mali

2017

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Chair

University of California, San Diego

2017

EPIGRAPH

In me the tiger sniffs the rose

—Siegfried Sassoon

TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE THESIS

Dynamic Genetic Control:
Manipulating Cell Behaviors Using Heat

by

Yanmin Ji

Master of Science in Bioegnineering

University of California, San Diego, 2017

Professor Peter Yingxiao Wang, Chair

Although Chimeric Antigen Receptor (CAR) T cells have been proven to be powerful in eradicating tumors. However, its non-specific activity against normal tissues and cells has been a major problem. Here, utilizing the activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) under heat, the translocation of nuclear factor of activated T-cells (NFAT) triggered by Calcium influx, and the recombination effects of Cre-lox system, we have developed a system to remotely manipulate the expression of proteins in cells under the control of both heat and drugs. The significance of this project is to provide a method to precisely control the activities

of CAR T cells within targeted local tissues.

Chapter 1

Background

Chimeric antigen receptor (CAR) T cell therapy is a potential method to treat cancer, paticularly with the benefit of having the resultant central memory T cells that can last for years in suppressing the cancer relapse suppression [1]. This treatment engineers T cells with a synthetic CAR that control them to kill malignant cells. CAR-expressing T cells have been proved to have the potential to cure leukemia and other cancers [1]. However, one major problem raised from clinical trials is that the non-specific activity against normal, non-malignant cells can be life-threatening [2]. In fact, off-tumor toxicities against the lung, gray matter in the brain, and cardiac muscles, have been caused multiple cases of deaths [3, 4]. Therefore, there is an urgent need of a precise control system to control the effects of CAR T cell therapy within local tissues. Although cells can be remotely manipulated by multiple different stimulations, one key factor of the viability of cells is the length of exposure. Thus, the first step towards finding a precise way to control cells is to design an efficient control system that can deliver the external signal in a relatively short time. Aiming to shorten the time of stimulation, this project utilized several control components that have been demonstrated in mammalian cells, and combined them to dynamically control cells' response.

This section provides the essential background for all components of the control system used in this project: transient receptor potential cation channel subfamily V member 1 (TRPV1), nuclear factor of activated T-cells (NFAT), and Cre-Lox recombination system.

1.1 Heat-activatable channel TRPV1

In 1997, TRPV1 was discovered as an sensitive receptor for Capsaicin, the essential ingredient in 'hot' peppers. [5]. Sensory neurons expressing TRPV1 channels were observed to have significant calcium influx with the presence of Capsaicin. After transfecting human embryonic kidney 293 (HEK 293) cells with cloned TRPV1 channels, these cells were observed to be activated by sudden changes in temperature with an *in vitro* threshold of 43 °C. These suggests that TRPV1 channels might stay inactivated at normal temperature of human body (37 °C). However, further investigations need to be done to confirm the leakage at lower temperature. Besides noxious temperature and capsaicin, the signals/drugs that can activate/deactivate TRPV1 channels are still being discovered [6]: mild acidification [7]; bradykinin [8]; nerve growth factor [9]; anadamide [10]; arachidonic acid metabolites [11]; lipoxygenase products [12]; leukotriene B_4 [13]; prokineticins. [14].

Furthermore, molecular biology researches proved that TRPV1 is a putative six-transmembrane-spanning protein with a pore region between transmembrane segments 5 and 6 [6]. According to cation current across the membrane, TRPV1 is determined to be an non-selective cation channel but with a high calcium ion permeability [5]. The response of TRPV1 channel to stimulations has been shown to be dynamically complex and have the tendency of adapting or desensitizing with continuous stimulations, such as heat and capsaicin, in sensory neurons [15, 16]. This phenomenon of desensitizing, at

least partially, depends on the extracellular calcium ions [16, 17].

Besides TRPV1, some other members in transient receptor potential (TRP) family can also be activated by heat at different levels. For example, TRP subfamily ankyrin, member 1 (TRPA1) can be activated at temperature lower than 17 °C [18]; TRP subfamily melastatin, member 8 (TRPM8) can be activated at temperature lower than 23 °C [19]; TRP subfamily vanilloid, member 2 (TRPV2) can be activated at temperature above 53 °C [20]; and TRP subfamily vanilloid, member 3 (TRPV3) can be activated at temperature above 33 °C [21]. Considering the ultimate goal of this project is to manipulate cells' *in vivo* behavior, the threshold of the chosen channel should be slightly above the routine temperature, but absolutely lower than extremely noxious temperature. Therefore, TRPV1 was considered as the primary choice for the project.

1.2 Nuclear translocation of NFAT

NFAT refers to a group of transcription factors that plays essential roles in immune system. Although first discovered in activated T cells, different NFAT family members have been identified in all cell types that have been investigated. NFAT family current has five members: NFAT1 (or NFATc2), NFAT2 (or NFATc1), NFAT3 (or NFATc4), NFAT4 (or NFATc3) and NFAT5. While NFAT5 is regulated by osmotic stress [22], all other four NFAT members are controlled by calcium influx [23]. Also, all NFAT members except NFAT 3 are discovered in immune system. Researchers have demonstrated that the calcium flow from intracellular stores is not sufficient to stimulate the function of NFAT [24], proving that NFAT should be relatively tightly regulated by calcium influx across the cell membrane. The molecular construct of NFAT suggests that NFAT can shuttle between the inside and the outside of the nucleus because of its nuclear localization signal (NLS) and nuclear exportation signal (NES). It is the balance of these two signals

that induces the shuttling of NFAT across the nuclear envelope.

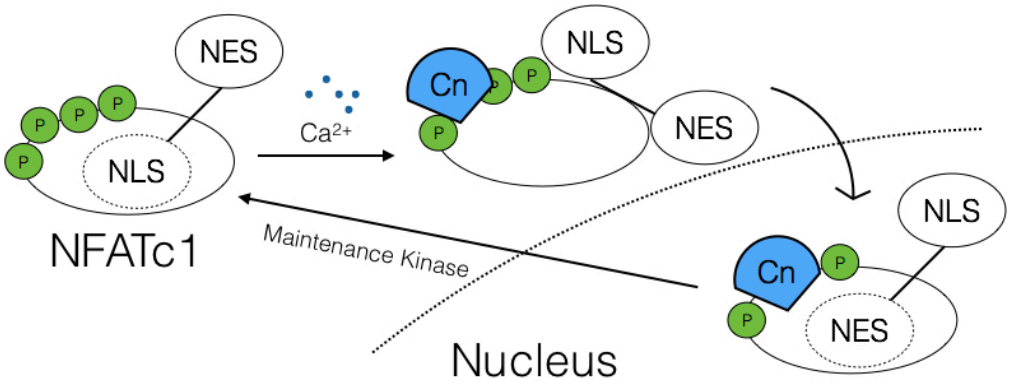


Figure 1.1: Calcium ions trigger the nuclear translocation of NFATc1: when NES is exposed, NFATc1 is kept out of the nucleus. With the presence of Calcium influx, calcineurin (CaN) will be activated and dephosphorylate NFATc1 to induce the conformational change, which can expose NLS of NFATc1. After NLS is exposed, NFATc1 will translocate into the nucleus. Then, with the help of maintenance kinase, NES will be exposed again, leading NFATc1 to shuttle back to the cytoplasm.

Calcium influx is essential to the translocation of NFAT because of calcineurin (CaN), a calcium-dependent serine-threonine phosphatase. The influx of calcium ions can activate calmodulin (CaM) by having calcium ions binding to the four calcium binding sites of CaM. Then, CaM can bind to the regulatory domain (RD) of CaN, and introduce a change in conformation, which pushs an autoinhibitory domain (AID) out of the active site, and activates the function of CaN [25]. The activated CaN, in turn, rapidly dephosphorylates the SP-repeats and the serine-rich region within the N terminus of the cytoplasmic components (NFATc proteins) [26]. The dephosphorylation of NFATc proteins leads to some conformational changes, and exposes its NLS for recognition, which induce the nuclear translocation of the NFATc protein, as shown in Figure 1.1. After translocating into the nucleus, if the NFATc protein is phosphorylated again, another conformational change will happen to expose its NES, and translocate the protein back into the cytoplasm, stopping any NFAT-mediated transcription. It needs to be mentioned that maintenance kinases and export kinases also help in regulating the activities of NFAT.

Maintenance kinases help to keep NFATc proteins out of the nucleus prior to calcium influxes, while export kinases help to export NFATc proteins after their phosphorylation [27].

1.3 Cre-lox recombination system

Cre-lox recombination system is a site-specific gene editing system that utilizes Cre recombinase, and can be used for inversions, deletions and translocations. Cre-lox recombination system have been applied in various different fields after its discovery: from inducing gene expression in mammalian cells [28], editing DNA sequence in developing T cells [29], to generating site- and organ-specific mouse lines [30].

Cre recombinase is a 38-kDa tyrosine recombinase enzyme first isolated from P1 Bacteriophage. It is believed to be essential in the process of rearranging genome after DNA replication in P1 [31]. The enzyme achieves site-specific gene editing using a mechanism similar to that of type I topoisomerase. As illustrated in three-dimensional structural data, Cre recombinase has a divergent helical N-terminal, and a more uniform and relatively helical C-terminal, forming a C-shaped site for the binding of the enzyme and DNA. When the enzyme recognizes two lox sites on the target sequence, it can modify the gene based on the relative positions and orientations of these two lox sites.

There are three possible situations when Cre recognizes two lox sites as shown in the Figure 1.2: first, if two lox sites are on the same DNA sequence, and have the same orientation, then anything between two lox sites will be deleted as circular DNA. Second, if two lox sites exist on the same DNA sequence, but have opposite orientations, then the sequence between two lox sites will be inverted. Lastly, if two lox sites are on different DNA sequence, but same orientation, then one half of the first sequence will switch with the same half of another sequence.

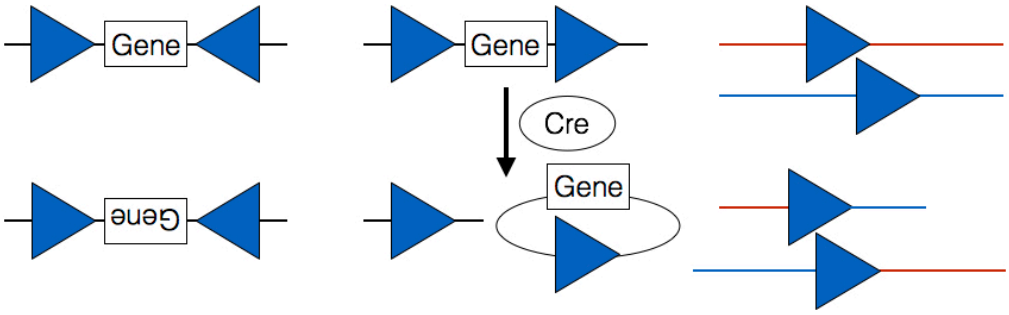


Figure 1.2: Different scenarios of Cre-lox recombination system: (from left to right) inversion, deletion and translocation. Blue triangles are lox sites with different orientations

In order to improve the specificity and efficiency of Cre-lox system, researchers have modified the system in three ways: translocation of Cre, spliting Cre, and combination of different lox sites. A typical application of the translocation of Cre is its combination with estrogen ligand-binding domain (ER^{T2}) [32]. The fusion protein Cre-ER^{T2} stays out of the nucleus without any stimulation. With the presence of Tamoxifen, Cre-ER^{T2} will translocate into the nucleus, and have Cre functioning with lox sites. Another way to improve the performance of Cre is to split intact Cre into two halves. For example, Kirchhoff et al. constructed Cre 19-59 and Cre 60-343, and fused both fragments with the constitutively dimerizing coiled-coil leucine zipper domain of GCN4 to facilitate the association of two constructs[33]. Furthermore, different combinations of various lox sites have been proved to have effects on the function of Cre.

Chapter 2

Results

2.1 Initial Design

TRPV1 channel, as it can be activated at 43 °C, which is significantly higher than the regular human body temperature, was used as the main control of our system. Since TRPV1 has higher sensitivity to Calcium than that to any other ions, NFATc1, nuclear translocation of which depends on Calcium influx, was fused to control the localization of Cre. Lox-containing reporter loxH-ZsGreen-STOP-loxP-mCherry (Cre Stoplight) should stay within the nucleus. At basal level, as shown in Figure 2.1, Cre-NFATc1 will stay out of the nucleus, separating Cre and lox sites and having no recombination. At this time, Cre Stoplight should constitutively express ZsGreen. After heat is applied, TRPV1 channel will introduce Calcium influxes, activating the nuclear translocation of NFATc1, which will bring Cre together into the nucleus. Thus, Cre can recognize lox sites on Cre Stoplight, delete ZsGreen-Stop part, and convert ZsGreen signals to mCherry signals.

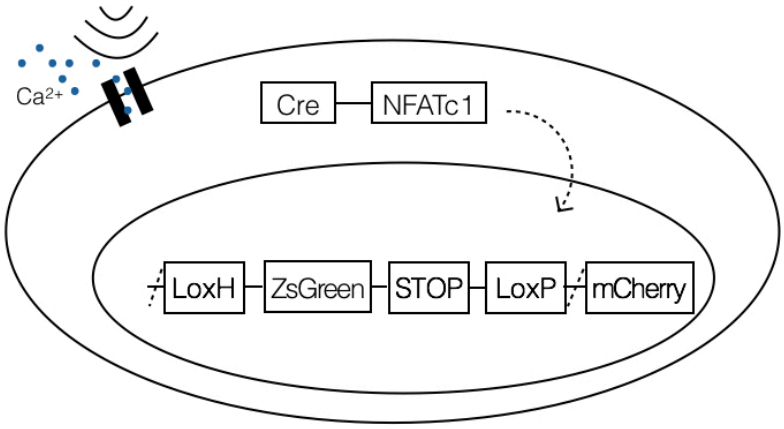
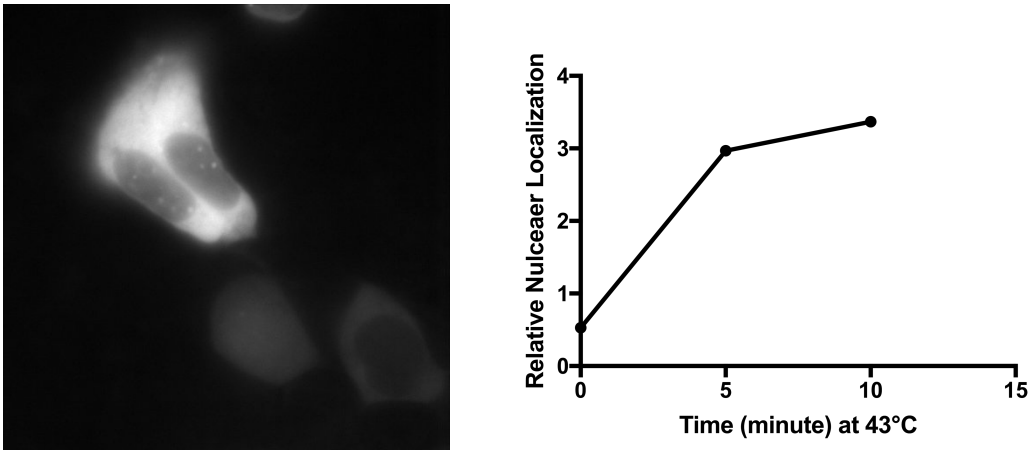


Figure 2.1: Schematics of Cre-NFATc1: at basal level, without heat, the construct Cre-NFATc1 stays out of the nucleus, the reporter stays in the nucleus, and the cells constitutively express ZsGreen proteins; after heat is applied, Ca^{2+} influx will be introduced by TRPV1 channels. Ca^{2+} ions, in turn, will activate nuclear translocation of NFATc1, which is fused to Cre. After Cre is pulled into the nucleus by NFATc1, it should recognize two lox sites on the reporter construct, and cut out the gene in between two lox sites, leading cells to change color from green to red

2.1.1 Characterizing single elements

Before constructing different constructs, the minimal concentration required for each single component of the control system was tested. Enhanced Green Fluorescent Protein (EGFP) was fused to NFATc1 proteins, and expressed in HEK 293T cells to verify its translocation. By using Ionomycin, an ionophore that is generally used to increase the intracellular level of Calcium (Ca^{2+}), NFATc1 was observed to start shuttling into the nucleus right after the addition of 1 µM the drug, and stay inside the nucleus for at least 20 minutes. The relative nuclear localization increased about twice after the treatment. Then, the fusion protein between EGFP and NFATc1 was tested coupled with TRPV1 channel to determine its feasibility of controlling by heat. As shown in Figure 2.2, although the background is relatively high, the increase of nuclear localization was significant as the heat time increases. Then, the heterodimerization of FKBP/FRB was tested together with Cre 19-59 (FKBP-CreN) and Cre 60-343 (FRB-CreC) respectively.



(a) (b)

Figure 2.2: Nuclear translocation of EGFP-NFATc1 under under heat: (a) shows the typical GFP-channel fluorescence imaging before any heat; (b) shows the change in relative nuclear localization as exposure time increases.

2.1.2 Engineering intact Cre with NFATc1

To keep the construct similar, we fused Cre and NFATc1 with the same linker from the fusion protein of EGFP and NFATc1 (SGLRS). After treating cells with heat for different time lengths, the mCherry signal in cells had no difference from the signal from the background, as shown in Figure 2.3. Therefore, we suspected: (1) the efficiency of our Cre-lox system was so low that the signal could not be captured; (2) the linker was too short that NFATc1 interfered the activity of Cre. Since we have demonstrated that FKBP-CreN and FRB-CreC can work fine with the presence of AP21967, and the efficiency of separated Cre is expected to be lower than that of intact Cre, the scenario (1) should not be the problem. Therefore, we started to optimize the linker between Cre and NFATc1. Since we have previously demonstrated the function of FKBP/FRB-CreN/CreC and Cre-ER^{T2}, we then tested whether the linkers from these two constructs could be used for the fusion of Cre and NFATc1. The linker from FKBP-CreN (ASPSNPGASNGS) and the linker from Cre-ER^{T2} (RLLEDGDLEP) were used to construct Cre-L1-NFATc1 and Cre-L2-NFATc1 respectively. The *in vitro* performance of these two constructs was

shown in Figure 2.4 While the recombination signal from Cre-L1-NFATc1 was still similar to the background, the signal from Cre-L2-NFATc1 was significantly higher than the background. However, it should be noticed that although the recombination efficiency of Cre-L2-NFATc1 was guaranteed, the leakage of signal was so high that the difference between the negative control group (no heat) and the experimental group was hard to determine. Therefore, we proposed to include FKBP/FRB as another layer of control into the system.

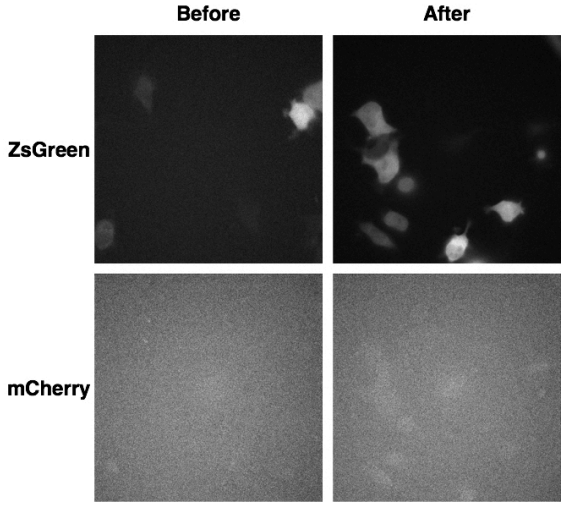


Figure 2.3: *in vitro* performance of Cre-NFATc1: the mCherry signal from Cre-NFATc1 after recombination was so weak that it was possible to be auto-florescence.

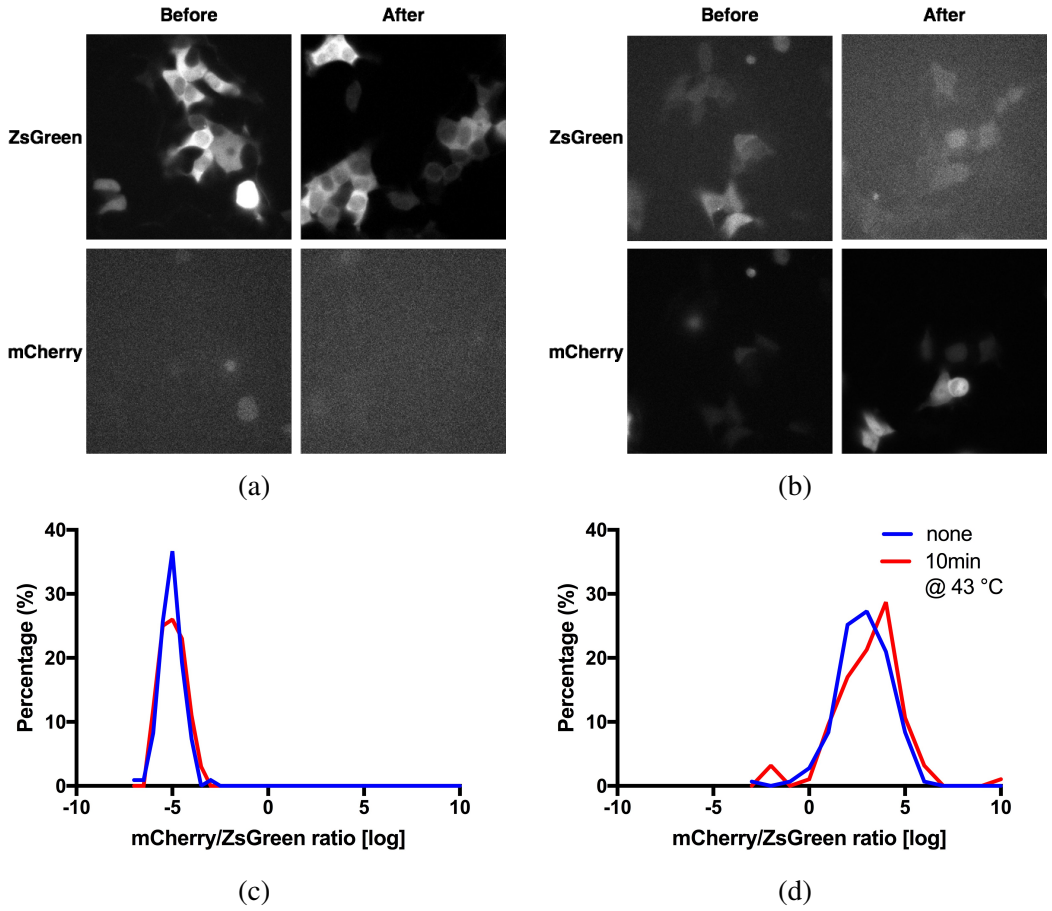
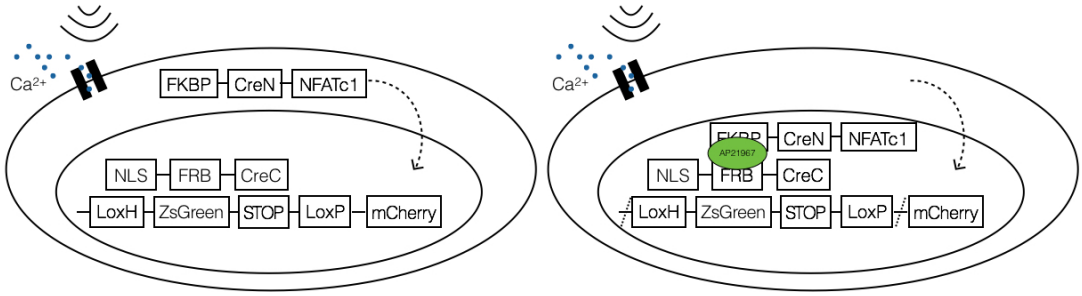


Figure 2.4: Comparison between different Cre-NFATc1 constructs: blue line: basal level, red line: after 10 minutes heat at 43 °C. As shown from (b) and (d), the mCherry signal was still weak with Cre-L1-NFATc1. For Cre-L2-NFATc1, from (c) and (e), although the background at the basal level is much higher than other constructs, the change between basal level and the experimental group is more significant than others.

2.2 Improved Design

Considering the fact that the combination of TRPV1 channel and NFATc1 has severe leakages even at the basal level, we started thinking to have another layer of controlling. The heterodimerization of FKBP and FRB, which is a great example of chemically induced dimerization (CID) system, was adapted into our system to provide a more precisely controlling. Discovered in 1996, FKBP and FRB can bind to each other

only with the presence of Rapamycin, a small molecular drug, or AP21967, a derivative of Rapamycin[34]. Therefore, ideally, at the basal level, FKBP-CreN-NFATc1 should stay out of the nucleus, and cells should solely express ZsGreen signal, as shown in Figure 2.5a. Then, with temperature increases to 43 °C, NFATc1 should be translocated into the nucleus. With no presence of Rapamycin, FKBP and FRB, as well as CreN and CreC should be separated, leading cells to express ZsGreen protein only. However, if we add Rapamycin after the temperature increases, FKBP and FRB will bind to each other, pulling CreN and CreC to form the intact Cre, in the nucleus. Therefore, we can observe the change in colors only if we apply both heat and Rapamycin on the cells, as shown in Figure 2.5b.



(a) (b)

Figure 2.5: Schematics of FKBP-CreN-NFATc1 and NLS-FRB-CreC: at the basal level, FKBP-CreN-NFATc1 stays out of the nucleus, while the reporter construct and NLS-FRB-CreC are constrained in the nucleus, and the cell should only have green signal (ZsGreen), as shown in (a). After heat is applied, FKBP-CreN-NFATc1 will be translocated into the nucleus. However, because of the absence of Rapamycin, FKBP and FRB cannot bind to each other, meaning the intact Cre cannot be formed. After the addition of Rapamycin, FKBP and FRB will bind together, pulling CreC and CreN to form the intact Cre, and, thus, Cre can function with lox sites to complete deletion, changing color from green to red (mCherry), as shown in (b)

2.2.1 Utilizing FKBP/FRB with split Cre

As shown in Figure 2.7, comparing with Cre-L2-NFATc1, FKBP-CreN-NFATc1 working together with NLS-FRB-CreC can have significant color change after the treat-

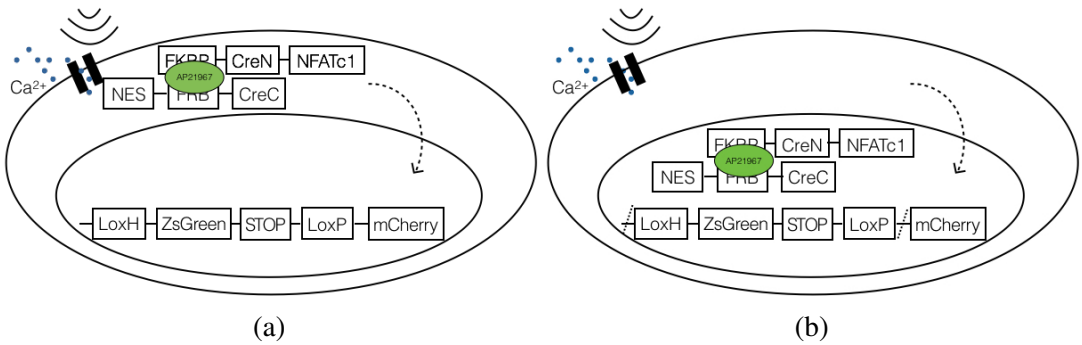
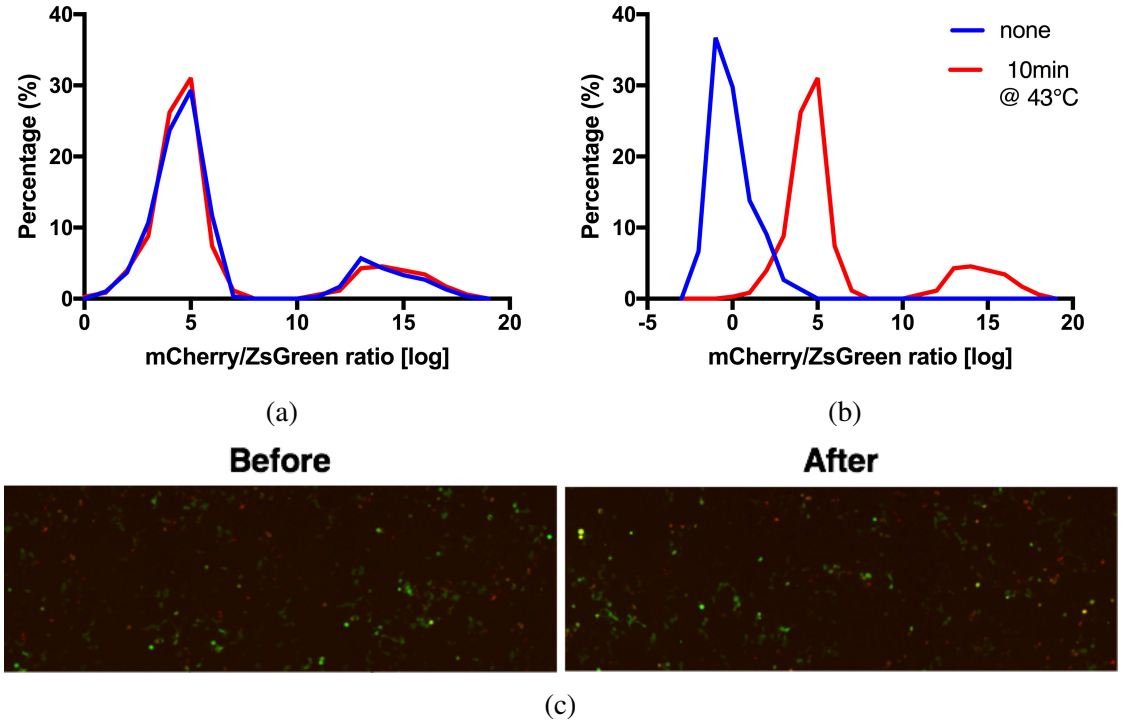


Figure 2.6: Schematics of FKBP-CreN-NFATc1 and NES-FRB-CreC: at the basal level, FKBP-CreN-NFATc1 and NES-FRB-CreC both stay out of the nucleus, while the reporter construct is in the nucleus, and the cell should only have green signal (ZsGreen), as shown in (a). After Rapamycin is added, FKBP and FRB should bind to each other, pulling CreC and CreN to form the intact Cre. Then, when heat is applied, the whole construct can be translocated into the nucleus. The intact Cre can now function with lox sites to complete deletion, changing color from green to red (mCherry), as shown in (b)

ment of heat and drug. However, it was noticed that even with Rapamycin only but no heat, majority of transfected cells can already express mCherry proteins, as in Figure 2.7c. The possible reason might be: (1) the leakage of TRPV1 allows the shuttling of NFATc1 at the basal level; (2) nonspecific FKBP/FRB heterodimerization allows the binding of Cre without Rapamycin; (3) the leakage of NFATc1 leads to uncontrolled translocation of NFATc1. In scenario (1), it was reported previously that the activation of TRPV1 channels is not strictly regulated [6]. However, cells transfected with FKBP-CreN-NFATc1 and NLS-FRB-CreC did not show any difference by culturing them at 33 °C and 37 °C. For scenario (2), previous examination of NLS-FKBP-CreN and NLS-FRB-CreC also showed minor leakages, but not as severe as the one fused with NFATc1. Therefore, the main focus was to work on the scenario (3) to keep FKBP-CreN out of the nucleus before any treatment. To reduce the power of NLS contained in NFATc1, another NES was inserted at the front of FKBP-CreN-NFATc1 or to replace the NLS in NLS-FRB-CreC. For groups containing NES-FRB-CreC, the workflow is different from other constructs: Rapamycin needs to be added first to form the heterodimer between

FKBP and FRB, then heat is applied to translocate the two constructs together, as shown in Figure 2.6. Two NES signals were selected because they were neither too strong to affect the translocation, nor too weak to have no effect: a relatively strong (nuclear factor kappa-light-chain-enhancer of activated B cells) NFkB inhibitor alpha (NFkBI) and a relatively weaker truncated 9cAMP-dependent protein kinase) PKI inhibitor alpha (PKIt) NES. Besides, different combinations of NESs were tested to find the most optimal solution.



(a) (b)

(c)

Figure 2.7: Comparisons between Cre-L2-NFATc1 and FKBP-CreN-NFATc1 + NLS-FRB-CreC: blue line: basal level, red line: after 10 minutes heat at 43 °C. From (a), the difference between the control group and the experimental group was hard to recognize for Cre-L2-NFATc1. However, from (b), the experimental and the control group were separated for FKBP-CreN-NFATc1+NLS-FRB-CreC. (c) shows fluorescence images for FKBP-CreN-NFATc1 + NLS-FRB-CreC. Left: before any heat or drug; right: after the addition of AP21967 and 10mins at 43 °C

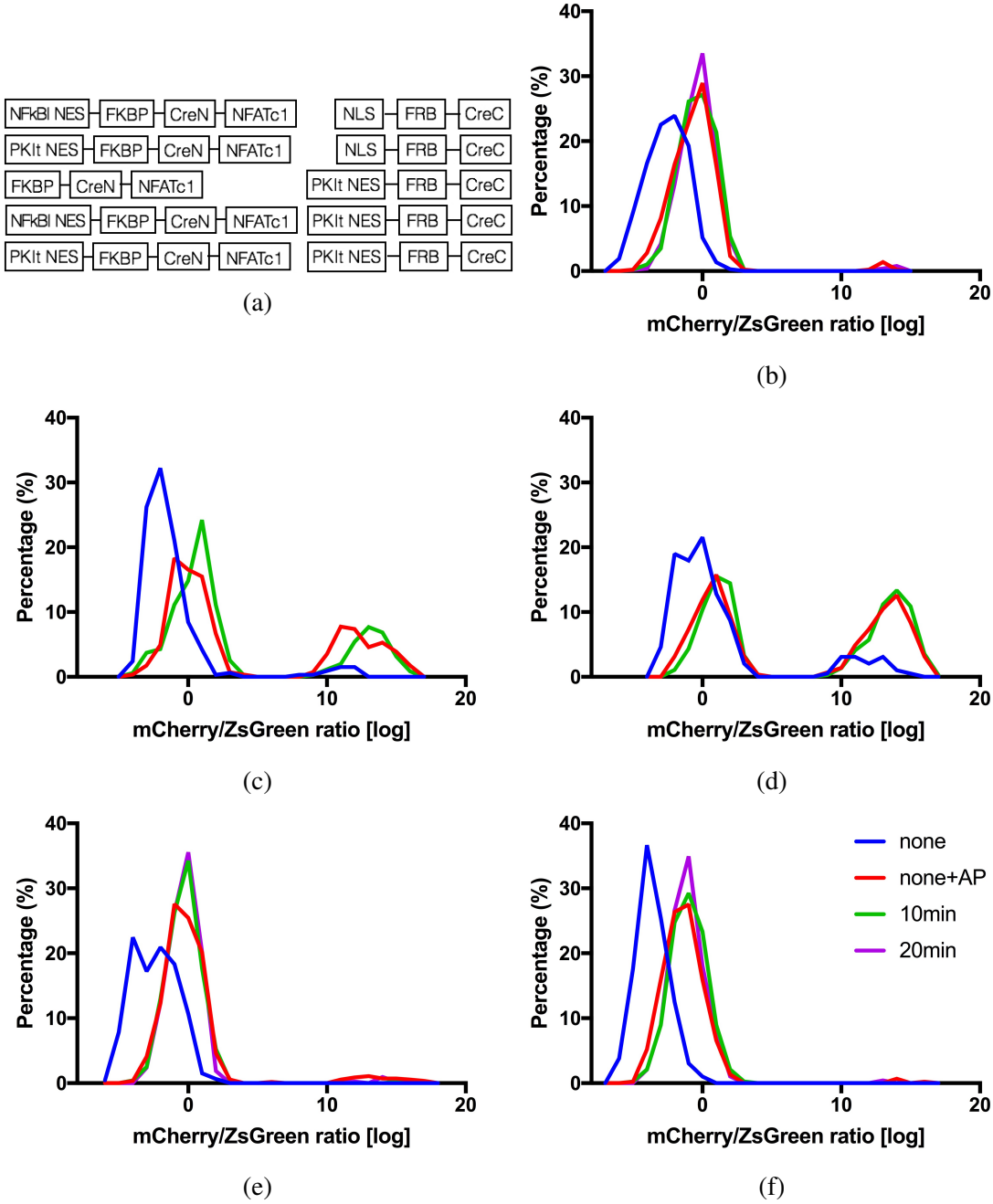


Figure 2.8: Comparisons between different fusions of NESs and FKBP-CreN-NFATc1 + FRB-CreC: Fig (a) shows all constructs used for comparison, with each row as a group. Fig (b) NFkBI NES-FKBP + NLS-FRB; Fig (c) PKIt NES-FKBP + NLS-FRB; Fig (d) FKBP + PKIt NES-FRB; Fig (e) NFkBI NES-FKBP + PKIt NES-FRB; Fig (f) PKIt NES-FKBP+PKIt NES-FRB

Chapter 3

Discussion

Leakage is one of the most important concerns of this project, mainly because Cre-lox recombination is irreversible, meaning that low-level leakage may result in significant noises over time. In the process of characterizing single elements for the initial design, it is noticed that although most of cells has dimmer signal from EGFP-NFATc1 within the nucleus than that from the outside of the nucleus, significant leakages are still observed before any treatment. The possible reasons are: (1) since Calcium ions are broadly used for signaling and other activities within cells, it is possible that the spontaneous Calcium ions influxes within the cells can induce the translocation of NFATc1; (2) NFATc1 itself is not tightly regulated, which means even there is no Calcium ion influx, some of NFATc1 proteins will still shuttle across the nuclear membrane. The scenario (1) is still hard to determine, mainly because detaching or washing cells will possibly disturbing their balances of Calcium ions. The scenario (2) can be easily solved by adding additional stronger NES sequence in front of the construct, which has been done in this project. It is possible that the additional NES is too strong that the NLS within the NFATc1 protein cannot function normally. However, it can be predicted that an appropriate NES can keep the balance just fine, so that it can both alleviate the leakage

of NFATc1 and allow some extents of NLS to translocate NFATc1 into the nucleus. Also, the leakage of TRPV1 is noticed at the temperature below 43 °C. This phenomenon can be predicted as the threshold of TRPV1 channel, instead of an exact temperature, is a broad range of temperature. In fact, Davis et al. have reported that TRPV1 channels can be activated by temperatures from 33 to 54 °C [35]. Therefore, it might be useful to keep cells at lower temperature prior to any treatment to keep lower background. Also, considering the leakage at temperatures lower than the threshold, another TRP family proteins with higher threshold temperature can be used to partially activated at a relatively high temperature. Additionally, the leakage from the heterodimerization of FKBP/FRB exists, but the magnitude is so small that it can be neglected for now.

In the combination of FKBP-CreN-NFATc1 and NLS-FRB-CreC, although the significant background can be observed from no heat with AP21967 group, the increase from negatively control group to the experimental group is still obvious. Considering the fact that drug, instead of heat, contributes the most to the control of the system, it is reasonable to predict that FKBP-CreN-NFATc1 is already translocated into the nucleus before any heat. This phenomenon echoes with the discussion regarding the leakages of single elements mentioned above, and proves that the leakage of NFATc1 should be the most severe factor. Transfected cells have also been cultured at different temperatures (e.g. 30 °C and 33 °C) to examine if TRPV1 contributes to the leakage of NFATc1, and the results shows that different culturing temperature does not significantly affect the translocation of NFAT. Therefore, the main focus should be on keeping NFATc1 out of the nucleus prior to any treatment.

In the final design, different NESs are examined to find the most optimal combination. From the result, it should be observed that all of them show different levels of difference between no heat with Rapamycin (AP21967) groups and experimental groups. From Figure 2.8, the peak ratio for experimental group s(applied both AP21967 and 43

°C temperature) is consistently larger than that for both no treatment group and no heat with AP21967 group, demonstrating that increases in temperature can be used to switch the gene expression from ZsGreen to mCherry. To determine which the best combination is, we mainly focus on the right peak on the plot, if there is any. For the peak on the left, which has lower average ratio, it is possible to the the mixture of real recombination signal and nonspecific cell activities (such as cell growth or cell morphology changes with the drug). However, for the right peak, by closely examining the raw imaging data, majority of the cells have real and high mCherry/ZsGreen ratio. Out of all these experimental groups, the combination of PKIt NES-FKBP-CreN-NFATc1 + NLS-FRB-CreC has the largest increase because it has the largest difference in the right peak between the drug only group and the heat group. Also, since the x axis is in log scale, it is expected that the difference between each group will be much more significant. Therefore, we conclude that we obtained the preliminary sucess on designing a high-precision control system for CAR-T cell activation.

In the future, much stronger NESs, such as human immunodeficiency virus type 1 Rev Protein (HIV) NES, can be tested to better control the nuclear translocation of NFATc1 proteins. Also, other TRP subfamily proteins can be tested in order to eliminate the leakage of TRPV1 channels at lower temperatures. If the leakage can be successfully limited, since all components of the system have been broadly used in animals by other researchers, this system can directly be tested on animal models by replacing fluorescence proteins of the reporter with other components, such as CAR.

In conclusion, based on the heat sensitivity of TRPV1 channels, nuclear translocation of NFAT proteins, heterodimerization between FKBP and FRB and Cre-Lox recombination system, we successfully build a control system to switch gene expression under heat simulations. Although leakage is still one of the most important concerns of the system, we have demonstrated that this issue can be alleviated by the addition of

NES in the constructs, and possible can be eliminated by stronger NESs. Therefore, the preliminary success of this control system has been demonstrated.

Chapter 4

Materials & Methods

Plasmids: The initial Cre-NFAT was constructed by replacing EGFP in EGFPC1-huNFATc1EE-WT. Cre-L1-NFATc1 and Cre-L2-NFATc1 were constructed by cloning Cre and NFATc1 separately and inserted together into a pcDNA vector. NLS sequence at the beginning of FKBP-CreN was deleted by primer prior to any design. FKBP-CreN-NFATc1 was constructed by cloning FKBP-CreN and NFATc1 separately and inserting both constructs in a pcDNA vector. Additional NESs were cloned in front of FKBP or FRB by primers. Different versions of the reporter, Cre Stoplight was constructed by cloning different pieces separately and combining in to a pcDNA vector. Because of multiple repeated pieces, all constructs were assembled using Gibson Assembly. The constructs were transformed into DH5α competent E. coli cells for DNA amplification. DNA was then extracted using Sigma-Aldrich GenElute^{TM} Plasmid Miniprep kit and verified by both digestion and sequencing.

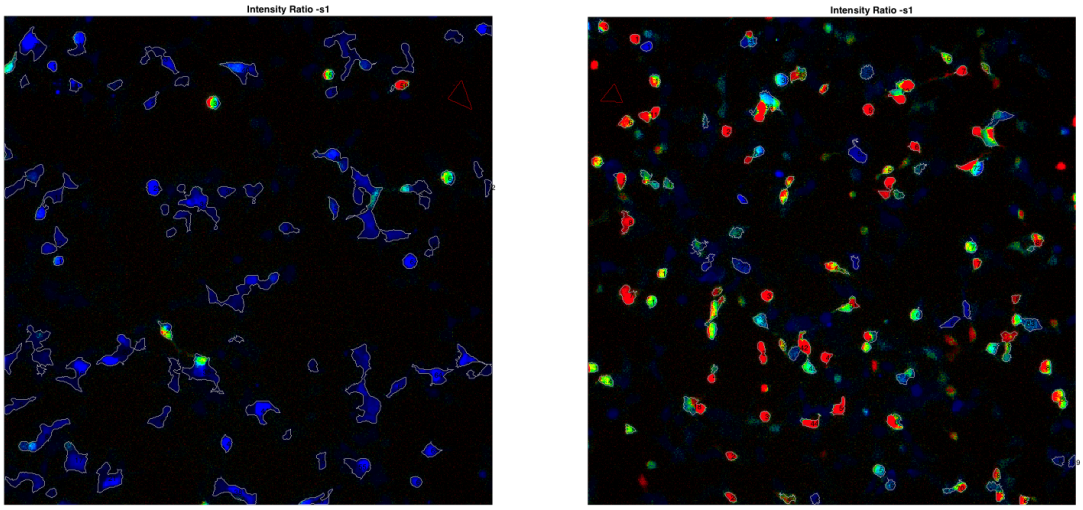
EGFPC1-huNFATc1EE-WT was a gift from Jerry Crabtree (Addgene plasmid # 24219). PcDNA_DiCre_59.F2 and pcDNA_DiCre_60.F2 were gifts from Jean-Paul Herman (Addgene plasmid # 60207 and # 60208). PVQ CMV NanoV1-2a-EGFP ferritin was a gift from Jeffrey Friedman (Addgene plasmid # 79649). Cre Stoplight 2.4 was a

gift from Thomas Hughes (Addgene plasmid # 37402).

Cell preparation: HEK cells were cultured in Advanced DMEM media (Gibco) added with 10% FBS, penicillin-streptomycin, and L-glutamine, transfected in transfection media (no penicillin-streptomycin added). Cells were cultured at 37°C in a 5%-CO2 and 95%-humidified incubator. Transfection was conducted with the Lipofectamine 3000 kit (Sigma-Aldrich) about 48 hours before imaging in transfection media. Experimental groups were treated about 16-24 hours before imaging. 500nM AP21967 (Clontech), a derivative of Rapamycin, and 1nM Ionomycin were also added right before the treatment, depending on the design of the experiment. Heat treatment was done by another 43°C 5%-CO2 and 95%-humidified incubator. For imaging, cells were then plated onto glass-bottom dishes (Cell E&G) coated overnight with fibronectin at 20 µg/mL concentration in culturing media 24 hours before imaging. For flow cytometry, cells were washed twice with PBS, detached with 0.5x trpysin-EDTA, neutralized with double-volume culturing medium, centrifuged with 1,000 rpm for 3 minutes, and resuspended in PBS right before the experiment.

Imaging acquisition and analysis: A Nikon Eclipse Ti inverted microscope installed with a 300 W Xenon lamp (Atlas Speialty Lighting), an electron multiplying (EM) CCD camera (QuantEM:512SC, Photometrics), and 100x, 40x and 10x DIC Nikon microscope objectives were used to capture all imaging data with the MetaMorph 7.8.8.0 software (Molecular Devices) or Metafluor 7.8.8.0 software (Molecular Devices). The microscope is equipped with a EGFP(FITC/Cy2) cube (#49002) and a mCherry/Texas Red cube (#49008) from Chroma Technology. Image analysis for all the acquired images was conducted on Fluocell, an image analysis software tool developed in the Wang Lab, and Quantify, an add-on of Fluocell to automatically analyze multiple cells within the same figure and calculate the fluorescence ratio, as shown in Figure 4.1.

Flow cytometry data acquisition and analysis: A BD Accuri C6 flow cytome-



(a) (b)

Figure 4.1: Fluocell can automatically detect and analyze single cells: Fluocell was used to analyze imaging data. The combination of mCherry channel and Zs-Green channel were used to detect cells, which were denoted by its white boundaries. MCherry/ZsGreen ratio was calculated and shown in color for each detected cell. The ratio from low to high was denoted as blue to red

ter was used to collect flow cytometry data. The flow cytometer has two excitation light: Blue (488 nm) and Red (640 nm). Two (533/30 nm and 610/20 nm)out of four emission filters were used for this project. All datas were analyzed using BD Accuri C6 software. Color compensation was done by adjusting channel intensity based on pure ZsGreen and mCherry proteins.

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